EXHIBIT B

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Flow cytometric analysis of platelet activation by different collagen types present in the vessel wall

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Summary. The interaction of platelets with collagens of the vessel wall is a critical event in primary haemostasis. Although numerous studies have examined the ability of various collagen types to support platelet adhesion, little is known concerning the relative ability of different collagens to elicit specific activation markers in platelets. In this report, flow cytometric analysis has been utilized to evaluate the ability of various native collagen types to elicit secondary activation events in human platelets. Collagen types I, III, V and VI induced α-granule secretion and up-regulation of cell surface glycoprotein (GP) IIb/IIIa. In contrast, collagen type IV did not elicit these responses in the concentration ranges examined. Dose–response curves for α-granule secretion induced by the various collagen types indicated that human type III and human type I collagens were less effective than human type V, human type VI and calf skin type I. In addition, the ability of these various collagens to activate GPIIb/IIIa to its ligand binding conformation was even more heterogenous with only human type VI and calf skin type I readily promoting this transition. These data demonstrate that flow cytometric analysis of collagen-induced platelet activation is feasible and that collagen-mediated α-granule secretion and membrane glycoprotein redistribution in human platelets are separate events from activation of GPIIb/IIIa.

Keywords: platelet activation, collagen, P-selectin, glycoprotein IIb/IIIa, flow cytometry.

Platelet adherence to collagen is recognized as a critical initial event for generation of a haemostatic plug (Sixma *et al*, 1997). The complexity of this interaction is emphasized by the presence of at least seven different forms of collagen within the vessel wall (van der Rest & Garrone, 1991) and reports of several different collagen receptors on platelets (Moroi & Jung, 1997). To dissect the multitude of possible interactions, investigators have examined platelet binding to various forms of immobilized collagen under conditions of both flow and stasis (for reviews see Sixma *et al*, 1997; Moroi & Jung, 1997; Kehrel, 1995). These studies suggest that most fibrillar forms of collagen will support adhesion, activation and aggregation of platelets to varying degrees (Moroi & Jung, 1997).

The primary collagen receptor on platelets is considered by many to be GPIaIIa (integrin $\alpha_2\beta_1$) which is present on the platelet surface at ~1000–2000 copies/cell (Saelman *et al*, 1994; Kunicki *et al*, 1993). However, there are data to suggest that GPIV (CD36) (Tandon *et al*, 1989), GPVI (Kehrel *et al*, 1998) and a 65 kD membrane protein (Chiang *et al*, 1997) are also involved with collagen binding. The data for each receptor include antibody inhibition studies, direct receptor isolation and/or absence of collagen-induced responses in patients lacking specific membrane receptors. Each receptor is assumed to be constitutively active although some potentiation of receptor affinity has been reported (Wilkins *et al*, 1996), and several investigators have suggested that full activation of platelets requires synergistic cooperation between different forms of collagen receptors (Keely & Parise, 1996; Clemetson, 1995).

The examination of platelet function has been greatly facilitated by the introduction of flow cytometric techniques which allow analysis of adhesive protein binding (Jackson & Jennings, 1989; Heilmann *et al*, 1994) and secondary events elicited by activation (Johnston *et al*, 1987; Michelson *et al*, 1994; Michelson, 1992). Particularly relevant among these secondary activation events are the redistributions of membrane glycoproteins (Nurden, 1997) and activation of specific receptors (Ginsberg *et al*, 1995). However, the collagen–platelet interaction has not been considered amenable to flow cytometric study due to the polymerization of collagen in neutral solution resulting in the formation of insoluble macro-aggregates (Williams *et al*, 1978). These aggregates are large enough to interfere with light-scatter

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analysis during flow cytometry (Wierwille *et al*, 1997) as well as to simultaneously interact with multiple platelets. One approach to this problem utilized by other investigators employs methylated collagens which are slow to aggregate and therefore can be used for flow cytometric analysis of the platelet–collagen interaction (Wierwille *et al*, 1997).

In this report we present a flow cytometric study of activation events in platelets induced by native collagens; this was accomplished by utilizing low concentrations of unmodified collagens under experimental conditions which minimized collagen aggregation. This methodology allows an examination of the ability of various collagens to elicit α-granule secretion, glycoprotein redistribution and glycoprotein receptor activation in human platelets. These data demonstrate that several collagen types mimic traditional strong agonists whereas others do not.

MATERIALS AND METHODS

Materials. Collagens type I (calf skin), type I (human), type III (human), type IV (human), type V (human), Sepharose CL-2B, FITC-goat-anti-mouse-IgM (FITC-GAMM) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St Louis, Mo. Collagen type VI (human) was obtained from Heyltex Corp., Houston, Texas. Fluorescein isothiocyanate (FITC) and NHS-biotin were obtained from Calbiochem, La Jolla, Calif. Phycoerythrin-labelled streptavidin (PE-SA) was obtained from Molecular Probes, Eugene, Ore. Monoclonal antibody G5 recognizes human P-selectin and was provided by Dr R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, Okla.; antibody AP2 against human GPIIb/IIIa (Pidard *et al*, 1983) was provided by Dr T. Kunicki, Scripps Research Institute, La Jolla, Calif.; antibody PAC-1 which recognizes activated GPIIb/IIIa (Shattil *et al*, 1987) was provided by Dr S. Shattil, Scripps Research Institute, La Jolla, Calif.

Buffers. BSGC: buffered saline-glucose-citrate, 129 mM NaCl, 13·6 mM Na_3citrate, 11·1 mM glucose, 1·6 mM KH_2PO_4, 8·6 mM NaH_2PO_4, pH adjusted with NaOH to either 6·5 or 7·3. ACD: acid citrate dextrose, 38·1 mM citric acid, 74·8 mM Na_3citrate, 136 mM glucose. PBS: phosphate buffered saline, 150 mM NaCl, 10 mM NaH_2PO_4, pH 7·4. Saline: 150 mM NaCl. HEPES: 100 mM HEPES, pH 7·5.

Human platelets. Informed consent was obtained in accordance with local Institution Review Board guidelines. 5 ml blood was drawn into 0·5 ml ACD and then diluted with 5 ml of room temperature BSGC, pH 7·3. Platelet-rich plasma (PRP) was prepared in 12×75 mm plastic centrifuge tubes filled maximally and centrifuged at 170 *g* for 8 min at room temperature. 2 ml of PRP were removed and applied to a 25×55 mm column of Sepharose CL-2B equilibrated with BSGC, pH 6·5. Isolated platelets were counted and diluted in BSGC, pH 7·3 to a cell count of 4×10^7/ml.

Collagen solutions. Collagens were dissolved at 1 mg/ml in 85 mM acetic acid overnight at 4°C. Stock solutions were prepared with a 1:5 dilution with water to yield a final collagen concentration of 200 μg/ml in 17 mM acetic acid; these optically clear stock solutions were stored at 4°C in glass tubes and were stable for at least a month. In one set of experiments calf skin collagen at 200 μg/ml in 17 mM acetic acid was centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant was then compared to the starting collagen solution for its ability to activate platelets utilizing the assays described below. Centrifugation did not affect the ability of collagen to mediate platelet activation, thereby indicating that there was no significant level of pre-formed collagen oligomers in these stock solutions. Further analysis of oligomerization after neutralization is discussed below.

Platelet activation markers. Binding of 5 μg/ml biotin-G5, an anti-P-selectin antibody, was used to monitor α-granule secretion by activated human platelets. GPIIb/IIIa redistribution was monitored with 5 μg/ml FITC-AP2 (Pidard *et al*, 1983). GPIIb/IIIa activation in human platelets was monitored with 5 μg/ml PAC-1 (Shattil *et al*, 1987) which was subsequently detected with FITC-GAMM.

Collagen activation of platelets. Reactions were performed in 17×100 mm polypropylene round-bottom culture tubes. For a final collagen concentration of 20 μg/ml, 50 μl of collagen (200 μg/ml stock) and 150 μl of 17 mM acetic acid in saline were added to reaction tubes and kept on ice until needed. Immediately before the assay was initiated, 250 μl of RT 100 mM HEPES, pH 7·5, with 2 mM $CaCl_2$ and any relevant antibody (e.g. G5 or PAC-1) was added followed by 50 μl of gel-filtered platelets. The reaction proceeded at 37°C for 10 min and was stopped with 4 ml of ice-cold 1% formalin in PBS. After 20 min of fixation at RT, 8 ml of 1 mg/ml BSA in PBS (BSA-PBS) were added, the platelets pelleted at 1500 *g* for 15 min, and the pellet resuspended in 400 μl BSA-PBS. 200 μl of the fixed platelets were transferred to 12×75 mm polypropylene tubes and labelled with the respective detection system, 5 μg/ml PE-SA for biotinylated antibodies, 10 μg/ml FITC-GAMM for PAC-1 or 5 μg/ml FITC-AP2 for GPIIb/IIIa. After 30 min of labelling at RT, the platelets were washed by addition of 3 ml of BSA-PBS and centrifuged at 1500 *g* for 15 min. The final pellet was resuspended in 800 μl of BSA-PBS for flow cytometric analysis.

Flow cytometry. Flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, San Jose, Calif.) utilizing ConSort 30 software. Platelets were identified by their characteristic forward scatter/side scatter or with platelet-specific antibodies (Pidard *et al*, 1983).

Flow cytometric analysis of collagen-induced changes was possible as a result of conditions which prevented any detectable collagen polymerization during the assay. These conditions included use of dilute stock solutions of collagen in weak acid, maintenance of low collagen concentrations after acid neutralization, and immediate utilization of collagen solutions upon neutralization as detailed above. With these experimental conditions, no collagen oligomers interfering with flow cytometric examination were observed utilizing forward light scatter/side light scatter analysis. Two additional controls were performed. First, collagen at 10 μg/ml was neutralized with HEPES buffer as described above and pre-incubated at 37°C for time periods ranging from 0 to 10 min before addition of platelets; no alteration in platelet response was observed. If collagen oligomerization upon neutralization were a significant contributor to the described

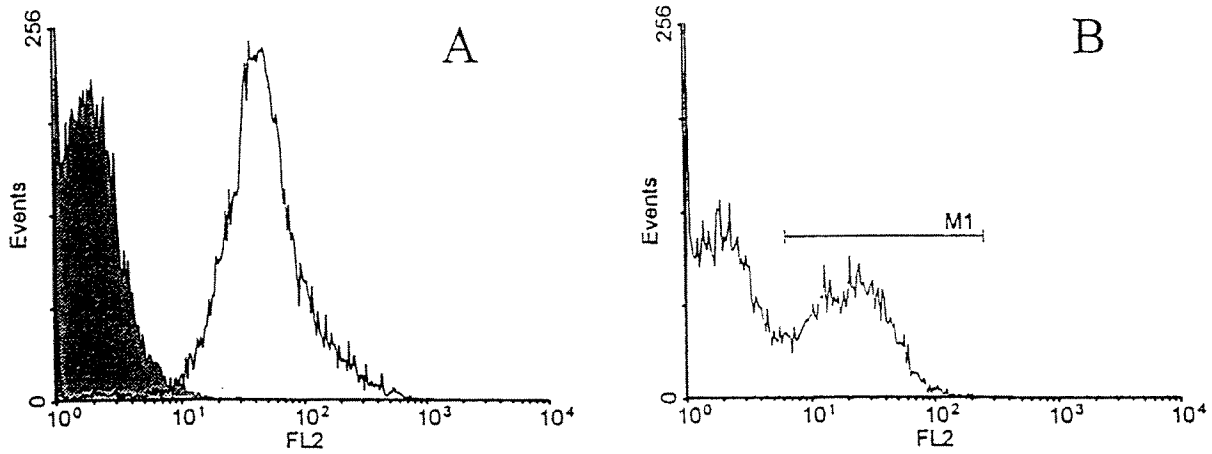

Fig 1. Collagen-mediated activation of human platelets. Panel A: calf skin collagen type I (10 μg/ml) was incubated with gel-filtered human platelets at 37°C. Alpha-granule secretion in response to collagen was monitored with an anti-P-selectin antibody (biotinylated G5); bound G5 was detected with phycoerythrin-streptavidin (PE-SA, FL_2, abscissa). The shaded area represents an FL_2 histogram for control platelets, and the solid line indicates the FL_2 histogram for collagen-activated platelets. Panel B: platelets were activated with an intermediate collagen concentration (1 μg/ml) and stained for P-selectin expression as detailed. Note that only 38% of the platelets (gate M1) responded to this sub-maximal collagen concentration.

activation events, a pre-incubation step would be expected to modify the platelet response. These data suggest that oligomerization during the time scale of these assays is not a significant variable. Secondly, possible collagen-induced aggregation of platelets was monitored by co-incubation with an equal number of washed erythrocytes. The ratio of platelets to erythrocytes before and after incubation with various collagen types was constant, indicating that no significant platelet aggregation occurred (data not shown).

RESULTS

Incubation of human platelets with either 10 μg/ml or 1 μg/ml of calf skin type I collagen resulted in expression of cell

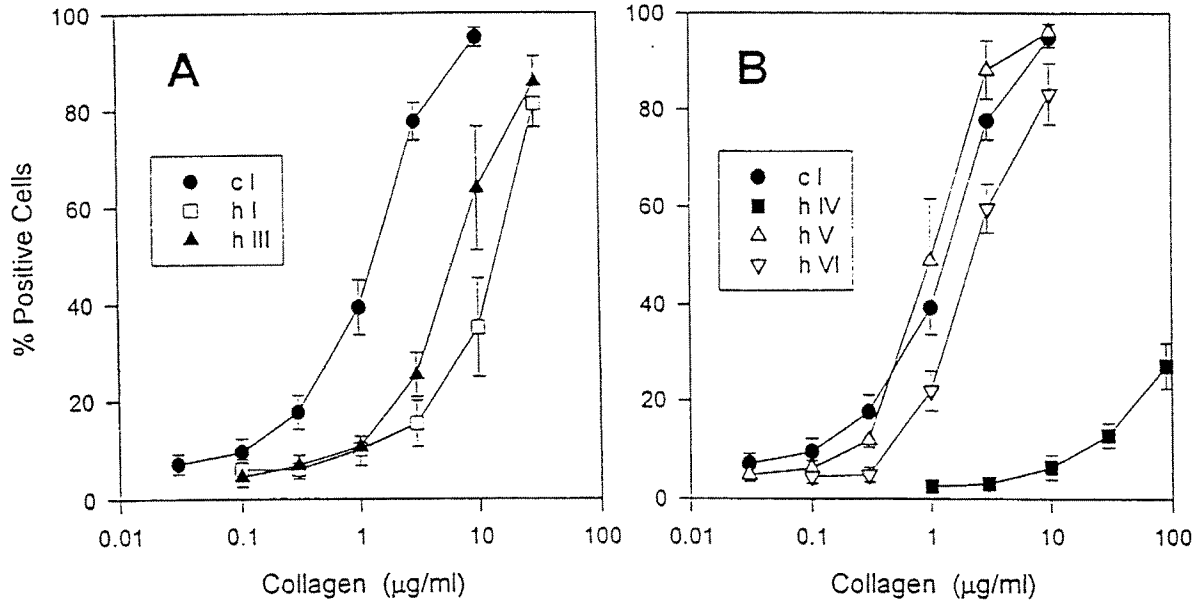

Fig 2. Dose–response curves for collagen stimulation of human platelets. Graded doses of various collagen types were reacted with gel-filtered, human platelets as detailed in Fig 1. The percentage of platelets positive for surface P-selectin was then quantitated (ordinate) and plotted versus collagen concentration (abscissa). Panel A represents calf skin collagen type I (solid circle), human type I (open square) and human type III (solid triangle); panel B represents calf skin collagen type I (solid circle), human type IV (solid square), human type V (up triangle) and human type VI (down triangle). Note that five of the six collagen types elicited significant α-granule secretion. Data represent mean ± 1 SD; $n = 3–6$.

surface P-selectin on 97% or 38%, respectively, of all cells (Fig 1). The response of platelets to collagen was further analysed with graded doses of each collagen type over a concentration range of 0·03–90 μg/ml (Fig 2). Of the six different collagens tested, calf skin type I, human type V and human type VI were the most active. Human type III and human type I clearly elicited α-granule secretion although with apparent EC_{50} values approximately 10-fold higher than that observed with calf skin type I. And finally, human type IV collagen was not appreciably active in the concentration range utilized here.

Strong platelet agonists are also known to result in a redistribution of GPIIb/IIIa to the platelet surface (Nurden, 1997). The effect of graded doses of calf skin collagen type I on the surface level of GPIIb/IIIa in human platelets is demonstrated in Fig 3. In panel A the mean fluorescence for FITC-AP2, an anti-GP IIb/IIIa monoclonal, demonstrates a collagen-dose–response curve similar to that for α-granule secretion. In panel B this response was further dissected in a dual-labelling experiment by measuring the mean FITC-AP2 fluorescence for P-selectin-positive and P-selectin-negative platelets at each collagen concentration. These data demonstrated that the mean FITC-AP2 fluorescence for the P-selectin-negative population remained constant at the pre-treatment level whereas the P-selectin-positive population had an elevated level of fluorescence. This observation suggests that GPIIb/IIIa redistribution occurred concurrently with α-granule secretion. The ability of various collagen types to induce redistribution of GPIIb/IIIa is shown in Fig 4. Similar to the results with P-selectin expression, collagen types I, III, V and VI elicited GPIIb/IIIa redistribution whereas type IV did not.

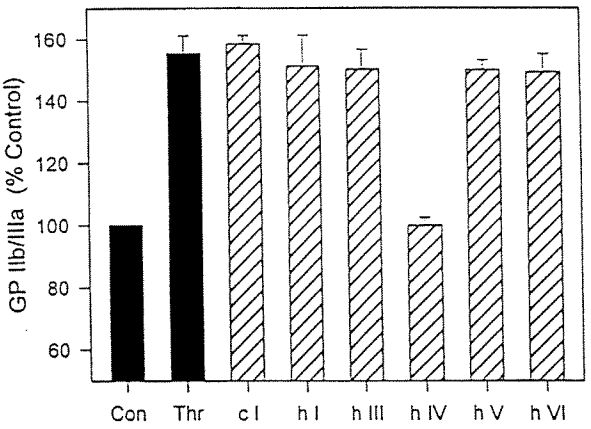

Fig 4. Effect of different collagens on GPIIb/IIIa redistribution. Gel-filtered human platelets were maximally stimulated with various collagen types and then stained with FITC-AP2. The mean fluorescence (ordinate) is shown for each collagen type (hatched bars); a quiescent control (Con) and thrombin-stimulated (Thr, 0·5 U/ml) sample are also shown for comparison (solid bars). Collagen types are identified by roman numerals; 'c' and 'h' signify calf and human, respectively. Data represent mean ±1 SD; $n = 3$.

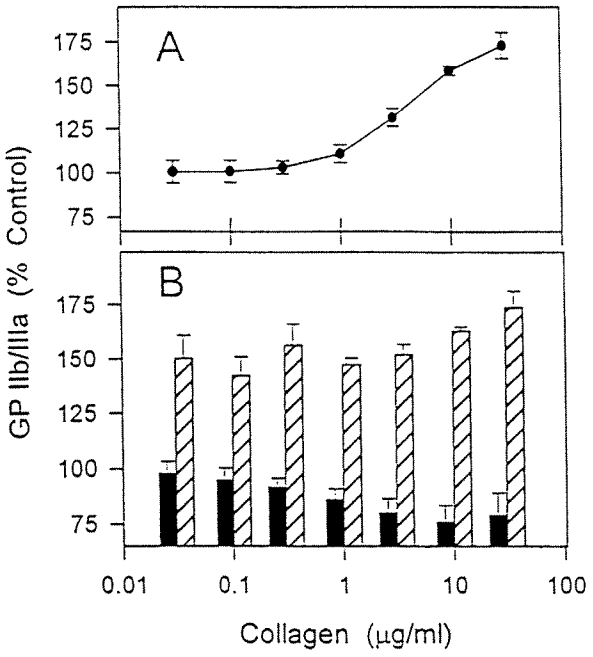

Fig 3. Effect of collagen activation on surface GPIIb/IIIa levels. Gel-filtered human platelets were reacted with graded doses of calf skin collagen type I and then labelled with FITC-AP2, an anti-GPIIb/IIIa monoclonal, and biotin-G5 to detect surface P-selectin. In panel A mean FL_1 fluorescence (FITC-AP2) for the total population is presented. Note the collagen-induced increase in surface exposed GPIIb/IIIa. In panel B the same samples depicted in the first panel were analysed for P-selectin expression, and the mean FL_1 for the P-selectin-positive (hatched bars) and P-selectin-negative platelets (solid bars) was determined. Note that the mean fluorescence for the GPIIb/IIIa antibody did not change from starting values for the P-selectin negative population whereas the P-selectin-positive population always had a high anti-GPIIb/IIIa fluorescence. Data represent mean ±1 SD; $n = 6$.

Fig 5 demonstrates that human type VI and calf skin type I were the most efficient in activating GPIIb/IIIa, whereas human collagen types I, III and V only activated GPIIb/IIIa at higher concentrations. Human type IV collagen resulted in essentially no change in PAC-1 binding. Even though there is no evidence that collagens directly bind to activated GPIIb/IIIa (De Groot & Sixma, 1997; Kehrel *et al*, 1998), there are reports that collagens can indirectly interact with this integrin (Coller *et al*, 1989). It was therefore necessary to consider the possibility that collagen might interfere with PAC-1 binding to activated GPIIb/IIIa. However, the fact that increased levels of these collagens potentiated PAC-1 binding (Fig 5) makes it unlikely that collagen was acting as a competitive inhibitor of PAC-1.

Activation of GPIIb/IIIa to its fibrinogen binding conformation is promoted by both strong (e.g. thrombin) and weak (e.g. ADP) agonists (Ware & Coller, 1995). Utilizing PAC-1, a monoclonal antibody which recognizes the activated conformation of GPIIb/IIIa (Shattil *et al*, 1987), we examined the ability of the various collagen types to activate human platelet GPIIb/IIIa. Fig 5 demonstrates that

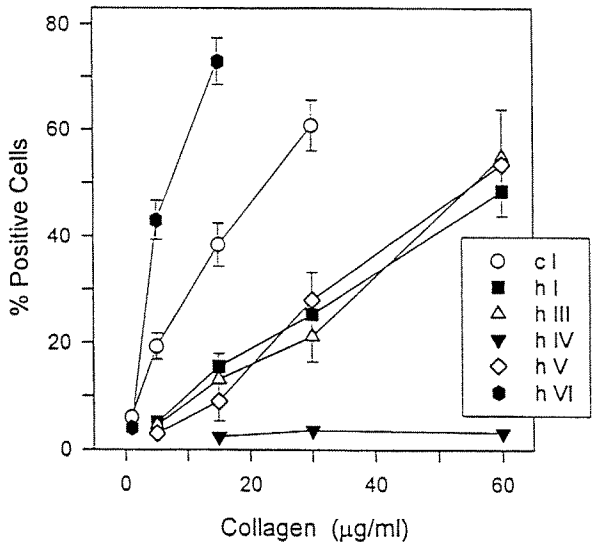

Fig 5. PAC-1 binding to platelets stimulated with collagen. Human platelets were activated with various collagen types and then analysed with PAC-1, an activation-dependent anti-GPIIb/IIIa monoclonal. The percentage of platelets positive for PAC-1 is indicated on the ordinate. The collagen types include: calf skin type I (open circle), human type I (solid square), human type III (open triangle), human type IV (solid triangle), human type V (open diamond) and human type VI (solid hexagon). Data represent mean ±1 SD; $n = 3$.

DISCUSSION

In vivo platelet adhesion to collagen exposed in ruptured blood vessels occurs under flow conditions, often at high shear. As a result, most experimental analyses of the collagen–platelet interaction have mimicked this natural process, utilizing artificial flow systems with variable shear rates (Sixma *et al*, 1997). Although these experiments are informative for many aspects of the collagen–platelet interaction, there are some parameters which do not readily lend themselves to analysis under such conditions. For example, the ability of various collagen types to promote glycoprotein redistribution as well as integrin activation is difficult to assess.

In this report we describe a technique which allows analysis of secondary activation events resulting from a fluid-phase interaction between collagen and platelets. With this system, collagens type I, III, V and VI elicited P-selectin expression and up-regulation of surface GPIIb/IIIa, although with different efficiencies for the individual collagen types. Human collagen type IV, in the concentration range examined, did not generate a significant response. Additionally, calf skin type I and human type VI collagens were the most efficient at eliciting activation of GPIIb/IIIa to its ligand binding conformation (Fig 5); however, higher collagen concentrations were generally required for GPIIb/IIIa activation than for either α-granule secretion or up-regulation of surface GPIIb/IIIa (Fig 2). The differential response of human platelets to the various collagen types is summarized in Table I. Although it may be tempting to compare the activating potential of these various collagens and their respective platelet-adhesive capabilities (Saelman *et al*, 1994), any correlation appears tenuous.

The different reactivities of the various collagen types may depend on one or more factors. (1) The individual collagen types may have different affinities for the relevant receptor. (2) Different receptors or sets of receptors may be responsible for responses with the various collagen types. (3) The collagens themselves may be structurally different with regards to the degree of oligomerization under the assay conditions utilized. Although it is clear that none of the collagens examined were sufficiently polymerized to interfere with flow cytometric analysis, we have not at this time conclusively addressed the question of oligomeric structure for each collagen type. However, ultracentrifugation of the collagen stock solutions did not affect their stimulatory properties, indicating that macropolymers did not form in the collagen stock solutions. In addition, a 10 min pre-incubation of neutralized collagen at the low protein concentrations utilized here did not modify the degree of platelet activation (see Methods). This finding is in agreement with a previous study examining the kinetics of collagen fibril formation which demonstrated a considerable lag time for polymerization at neutral pH (Williams *et al*, 1978).

The dose–response curves demonstrated in Fig 2 are strikingly similar to those observed with other agonists such as thrombin (Peng *et al*, 1994). The basis for differential reactivity within a platelet population is unclear (Thompson & Jabubowski, 1988). For thrombin, some of the variability

Table I. Summary of activation characteristics of several collagen types with human platelets.

	Agonist						
		Collagen type					
Activation parameter	Thrombin	Calf skin I	Human I	Human III	Human IV	Human V	Human VI
P-selectin expression	++	++	+	+	±	++	++
GPIIb/IIIa up-regulation	++	++	+	+	±	++	++
GPIIb/IIIa activation	++	++	+	+	−	+	++

The response of platelets to the various collagen types was monitored as detailed in Methods.

can be attributed to age differences (Peng *et al*, 1994); there are, however, other factors controlling reactivity which remain unknown regardless of the agonist.

Finally, the variation in concentration dependence for expression of different activation markers with a specific collagen type was unexpected. For example, type V collagen, which was among the strongest activators for eliciting P-selectin expression, was only a modest activator of GPIIb/IIIa. On the other hand, type VI collagen was able to elicit both changes at almost the same effector concentration. These observations indicated that α-granule secretion and GPIIb/IIIa redistribution were separate events from GPIIb/IIIa activation.

Activation events reported here represent the final steps of intracellular signalling pathways initiated by various collagens. Studies with other integrins have shown that receptor occupation can result in intracellular phosphorylation reactions culminating in platelet activation (Shattil *et al*, 1994), and occupation of the collagen receptor(s) is known to elicit a number of intracellular events, including phosphorylation of syk, src and PLCγ2 (Keely & Parise, 1996). The role of these events in expression of the secondary activation markers monitored here is not clear; however, it does appear likely that more than one receptor, and therefore multiple intracellular signalling pathways, are involved.

The current experiments have utilized a technique which allows a fluid phase interaction between human platelets and various collagen types. The strength of this procedure is the ability to utilize flow cytometry for analysis of secondary activation events resulting from collagen stimulation. Although this methodology has shed new light on the reactivity of the various collagen types, complementary experiments investigating the receptors and intracellular signalling utilized by the different collagens are required to fully elucidate the molecular basis for these differential reactivities.

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